Interleukin-6 Modulates the Expression of the Bone Morphogenic Protein Receptor Type II Through a Novel STAT3-microRNA Cluster 17/92 Pathway

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Abstract: Dysregulated expression of bone morphogenetic protein receptor type II (BMPR2) is a pathogenetic hallmark of pulmonary hypertension. Downregulation of BMPR2 protein but not mRNA has been observed in multiple animal models mimicking the disease, indicating a posttranscriptional mechanism of regulation. Because microRNAs (miRNAs) regulate gene expression mainly through inhibition of target gene translation, we hypothesized that miRNAs may play a role in the modulation of BMPR2. Performing a computational algorithm on the BMPR2 gene, several miRNAs encoded by the miRNA cluster 17/92 (miR-17/92) were retrieved as potential regulators. Ectopic overexpression of miR-17/92 resulted in a strong reduction of the BMPR2 protein, and a reporter gene system showed that BMPR2 is directly targeted by miR-17-5p and miR-20a. By stimulation experiments, we found that the miR-17/92 cluster is modulated by interleukin (IL)-6, a cytokine involved in the pathogenesis of pulmonary hypertension. Because IL-6 signaling is mainly mediated by STAT3 (signal transducer and activator of transcription 3), the expression of STAT3 was knocked down by small interfering RNA, which abolished the IL-6-mediated expression of miR-17/92. Consistent with these data, we found a highly conserved STAT3-binding site in the promoter region of the miR-17/92 gene (C13orf25). Promoter studies confirmed that IL-6 enhances transcription of C13orf25 through this distinct region. Finally, we showed that persistent activation of STAT3 leads to repressed protein expression of BMPR2. Taken together, we describe here a novel STAT3-miR-17/92-BMPR2 pathway, thus providing a mechanistic explanation for the loss of BMPR2 in the development of pulmonary hypertension.

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Interleukin-6 modulates the expression of the BMP receptor type II through a novel STAT3 - microRNA cluster 17/92 pathway

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Abstract

Dysregulated expression of bone morphogenetic protein receptor type II (BMPR2) is a pathogenetic hallmark of pulmonary hypertension. Downregulation of BMPR2 protein but not mRNA has been observed in multiple animal models mimicking the disease, indicating a posttranscriptional mechanism of regulation. Since microRNAs (miRNAs) regulate gene expression mainly through inhibition of target gene translation, we hypothesized that miRNAs may play a role in the modulation of BMPR2. Performing a computational algorithm on the BMPR2 gene, several miRNAs encoded by the microRNA cluster 17/92 (miR-17/92) were retrieved as potential regulators. Ectopic overexpression of miR-17/92 resulted in a strong reduction of the BMPR2 protein and a reporter gene system showed that BMPR2 is directly targeted by miR-17-5p and miR-20a. By stimulation experiments, we found that the miR-17/92 cluster is modulated by interleukin (IL-)6, a cytokine involved in the pathogenesis of pulmonary hypertension. Since IL-6 signaling is mainly mediated by signal transducer and activator of transcription (STAT)3, the expression of STAT3 was knocked down by siRNA, which abolished the IL-6-mediated expression of miR-17/92. Consistent with these data, we found a highly conserved STAT3 binding site in the promoter region of the miR-17/92 gene (C13orf25). Promoter studies confirmed that IL-6 enhances transcription of C13orf25 through this distinct region. Finally, we showed that persistent activation of STAT3 leads to repressed protein expression of BMPR2. Taken together, we describe here a novel STAT3 - miR-17/92 - BMPR2 pathway thus providing a mechanistic explanation for the loss of BMPR2 in the development of pulmonary hypertension.

Introduction

Pulmonary hypertension is a devastating condition defined by the sustained elevation of pulmonary vascular resistance that leads rapidly to right heart failure and death when left untreated.[1] The pathogenesis of pulmonary hypertension is characterized by vascular remodelling and vasoconstriction.[2] Many chemotactic and inflammatory factors have been associated with these vascular changes including interleukin (IL-)-6 and transforming growth factor (TGF)β.[3–5] In familial pulmonary arterial hypertension, germline mutations in the gene encoding the type II receptor of the bone morphogenetic protein (BMPR2) comprise a genetic hallmark of the disease.[6] BMPR2 is a surface protein receptor that belongs to the transforming growth factor (TGF) β family. Its expression on endothelial and vascular smooth muscle cells mediates binding of bone morphogenetic proteins (BMP) that have been identified as inhibitors of vascular smooth muscle cell proliferation while inducing cell death.[7] It thus was suggested that the downregulation of BMPR2 might lead to significant alterations in these signaling cascades and, ultimately, to remodelling of the pulmonary vascular bed.[8] Of interest, alterations in the surface expression of BMPR2 have also been described in non-genetic forms of pulmonary hypertension.[9] In addition, loss of BMPR2 has been observed in several animal models mimicking the disease.[8,10] The intracellular mechanisms leading to this downregulation however are yet to be elucidated. Studies focussing on the role of BMPR2 in the development of pulmonary arterial hypertension have observed reduced protein level of this surface receptor. Takahasi et al., for example, described the expression of BMPR2 in pulmonary arteries of rats under normal conditions and after exposure to hypoxic conditions. Thereby, hypoxia was found to reduce the expression of BMPR2 on the protein levels, whereas the levels of the corresponding mRNA were not affected adequately.[8] Similarly, in the monocrotaline-induced rat model of pulmonary hypertension, the expression of the BMPR2 protein was rapidly reduced without initial effects on the mRNA levels.[10]

These findings suggest a post-transcriptional mechanism, such as the involvement of microRNAs (miRNAs) that bind to their target mRNAs by Watson-Crick-base pairing at distinct seed regions and, thus, alter mRNA stability or affect protein translation.

An accumulating body of evidence suggests that up to one third of the human genome is regulated by miRNAs through post-transcriptional mechanisms.[11] Consequently, miRNAs have been associated with various cellular processes including cell death, differentiation and proliferation.[12] Based on computational algorithms (i.e. TargetScan) and the fact that surface protein receptors such as the TGFβ-receptor type II (TGFβR2) have already been shown to be regulated by microRNAs,[13] we identified the microRNA cluster 17/92 as potential modulator of BMPR2 expression. In the present in vitro study, we used human pulmonary arterial endothelial cells (HPAEC), hepatocellular carcinoma (HepG2) cells and human embryonic kidney (HEK)293 cells to address the following issues: (i) the role of miR-17/92 in the post-transcriptional regulation of BMPR2 expression; (ii) the effects of inflammatory cytokines and growth factors on the expression of miR-17/92; and, (iii) the role of the transcription factor signal transducer and activator of transcription (STAT)3 as master link between IL-6 and the modulation of BMPR2. Our data reveal for the first time a potential molecular mechanism explaining the downregulation of BMPR2 in the development of pulmonary arterial hypertension.

Materials and Methods

Cell culture. For cell culture, human embryonic kidney (HEK)293 cells and human hepatocellular carcinoma (HepG2) cells were used. Human pulmonary artery endothelial cells (HPAEC) were purchased from Cascade Biologics. All growth factors and stimulation agents (recombinant human IL-6, VEGF, PDGF) were purchased from R&D Systems.

Plasmid construction. For overexpression of the miR-17/92 cluster, genomic DNA encoding miR-17/92 was amplified and cloned into pcDNA3.1 + between the *Hind*III and *Eco*RI restriction sites as previously described.[14] Cells were transfected using Lipofectamine 2000.

Real time RT-PCR analysis. Total RNA was extracted using the RNeasy kit and quantification of specific RNA transcripts was performed by SYBR Green real-time PCRs, using the ABI Prism 7700 Sequence Detection System.

Quantification of mature microRNA-20a. Total RNA was extracted using the mirVana miRNA Isolation Kit. Mature microRNA-20a was detected by stem-loop reverse transcription followed by SYBR Green real-time PCR[15] and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase.

Western blot analysis. The following antibodies were used for Western blot: anti - human BMPR2, anti - human STAT3, anti - human Phospho-STAT3 and anti - α-Tubulin. Evaluation of the expression of specific proteins was performed by the Alpha Imager Software system via pixel quantification of the electronic image.

Reporter gene assay. A 1554 bp fragment of the 3'untranslated region (UTR) of BMPR2 was amplified out of genomic DNA. The PCR product was *Xba*I digested and cloned into the *Xba*I restriction site of the pGL3 control vector. As negative control, the anti-sense construct was used according to Kuhn et al.[16] HEK293 cells were transfected with the pGL3 control 3'UTR of BMPR2 "sense" or "anti-sense" construct. A vector encoding for the miR-17/92 cluster (pcDNA miR-17/92) and a vector for normalisation (pRL-SV40) were added. For inhibition of endogenous miRNAs, a similar protocol was applied with the use of anti-miRs. Luciferase

activity was measured using the Dual-Luciferase Reporter Assay System and the values obtained were normalized to the activity of Renilla luciferase.

Nuclear transfection. The endogenous expression of the human STAT3 gene was knocked down by validated siRNA for STAT3. Nuclear transfection of HPAEC was achieved by using the nucleofection kit HMVEC-L from Amaxa.

Statistics. For statistical analysis, GraphPad Prism Software was used.

All details are provided within the expanded Materials and Methods section in the online data supplement (http://circres.ahajournals.org).

Results

Computational analysis reveals BMPR2 as a target of the miR-17/92 cluster

Data from previous studies on the protein expression of BMPR2 in animal models of pulmonary hypertension suggested a post-transcriptional mechanism of regulation, possibly involving miRNAs. We thus performed a screening based on a computational algorithm (TargetScan, Whitehead Institute for Biomedical Research, www.targetscan.org) to identify distinct seed regions of miRNAs in the 3'untranslated region (UTR) of the BMPR2 gene. To improve prediction rates the focus was put only on highly conserved seed regions.[17] TargetScan retrieved several miRNAs for BMPR2, including miR-17-5p, miR-19a, miR-19b, miR-20a and miR-92. Of interest, these miRNAs are encoded by one single miRNA cluster, miR-17/92 located at the chromosome 13q31 in the 3rd intron of the C13orf25 gene.[18]

Overexpression of miR-17/92 reduces the expression of BMPR2

To verify the predicted miRNAs from the computational analysis on a functional basis, the entire cluster was cloned into a mammalian expression vector under the control of the SV40 promoter. Successful overexpression in HEK293 cells was confirmed by quantification of one representative mature microRNA derived from miR-17/92 (miR-20a) 72h after transfection. As shown in Figure 1a, the expression of miR-20a was significantly increased by 2.71 ± 0.45 fold ($p = 0.005$) as compared to the transfection with the empty vector (mock). The overexpression of the miR-17/92 cluster resulted in a significant decrease of BMPR2 on the protein level as analysed by Western blot (0.66 ± 0.06, $p = 0.002$, Fig. 1b). The time-dependent down-regulation is shown as supplementary Figure (Online Fig. I). The corresponding levels of mRNA were also significantly reduced (0.89 ± 0.06, $p = 0.03$, Fig. 1c). However, the changes observed on the mRNA level were much less impressive than the protein changes, indicating that the miRNA cluster represses the translation process of

BMPR2. The modest downregulation of mRNA levels, on the other hand, is a known phenomenon reflecting the interactive behaviour of miRNAs and mRNA.[19]

The reduction of BMPR2 is mediated directly by the action of miR-17-5p and miR-20a

In a next step, we addressed the question whether the observed reduction of BMPR2 protein is directly miRNA-driven. A part of the 3'UTR of BMPR2 (35 – 1589 bp) was cloned into the pGL3 control vector creating a luciferase reporter system with respective binding sites for the miRNAs 17-5p, 19a, 19b, 20a and 92 (Online Fig. II). The "anti-sense" construct was generated and employed as negative control.[16] Luciferase activity was assessed, and data were normalized to the Renilla luciferase activity. Co-transfection of the pGL3 3'UTR of BMPR2 "sense" construct and the miR-17/92 overexpressing vector yielded a lower relative luciferase activity as compared to mock when transfected into HEK293 cells (0.76 ± 0.12, Fig. 2a). The "anti-sense" construct was not affected by overexpression of the miR-17/92 cluster (1.06 ± 0.07). These findings imply a direct interaction between the 3'UTR of BMPR2 and the miRNAs derived from the miR-17/92 cluster. To identify the impact of the distinct miRNAs, each endogenous miRNA from the cluster was inhibited by the use of anti-sense RNA molecules (anti-miRs). As shown in Figure 2b, these blocking experiments revealed a significantly higher relative luciferase activity for anti-miR-17-5p (1.31 ± 0.21, $p = 0.03$) and anti-miR-20a (1.52 ± 0.24, $p = 0.008$), indicating a pivotal role of these two miRNAs in the interaction with the BMPR2.

The expression of miR-17/92 correlates with the activity of STAT3

A growing body of evidence suggests that the intracellular level of some miRNAs is regulated, at least in part, by the action of cytokines[20] and growth factors.[21] Since these factors have also been implicated in the pathogenesis of pulmonary hypertension[4] we stimulated

HPAEC with IL-6, VEGF and PDGF and quantified the expression of the preliminary transcript of miR-17/92 (C13orf25).

Figure 3a illustrates a significant upregulation of C13orf25 mRNA 30min and 1h after the stimulation of HPAEC with IL-6 (1.98 ± 0.19, p = 0.0018 and 1.87 ± 0.33, p = 0.045 respectively) and VEGF (1.96 ± 0.36, p = 0.045 and 1.58 ± 0.03, p = 0.001 respectively) as compared to unstimulated control cells. 4h after stimulation, the mRNA levels reached base line expression. The stimulation with PDGF did not affect the mRNA levels of C13orf25, probably due to the fact that HPAEC lack the receptor for PDGF (data not shown).

It was reported from previous experiments in endothelial cells, that IL-6 and VEGF share common signaling mechanisms including the STAT3 pathway.[22,23] STAT3 is a major transcription factor, which is shuttled from the cytoplasm into the nucleus upon activation by tyrosine phosphorylation of residue 705. When HPAEC were stimulated and analysed by Western blotting, a strong phosphorylation of STAT3 could be observed for IL-6 after 30 min, and, to a lesser extent, after 1h. However, no phosphorylation could be detected after the addition of VEGF (Fig. 3b). With respect to the data presented in Figure 3a, a correlation between activation of STAT3 and induction of the expression of C13orf25 can be postulated. According to this hypothesis, we performed siRNA experiments to knockdown the endogenous STAT3 expression in HPAEC. Thereby, as shown in Fig 3c, the expression of STAT3 could be reduced by 50%. Stimulation experiments after established siRNA-mediated reduction of STAT3 are summarized in Figure 3d. Interestingly, the stimulatory effect of IL-6 on the expression of C13orf25 was almost completely abolished by the functional knockdown of STAT3 as compared to stimulated scrambled control cells (p = 0.0027). Consistent with the Western blot (Fig. 3b), the difference observed between siRNA and scrambled transfected cells when stimulated with VEGF was statistically significant but considerably weaker than the difference upon stimulation with IL-6 (p = 0.0428). To evaluate whether stimulation of

HPAEC with IL-6 and VEGF might also affect the expression of mature miRNA derived from miR-17/92, expression levels of mature miR-20a were assessed and a significant upregulation was detected (1.44 ± 0.31, p = 0.033, Fig. 3e) as compared to control cells 24h after stimulation with IL-6. Expression levels of miR-20a measured after stimulation with VEGF however did not reach a statistically significant increase (1.44 ± 0.61, p = 0.187). These data highlight the role of IL-6 as an inducer of mature microRNAs derived from the cluster miR-17/92.

Identification of a highly conserved STAT3 binding site in the promoter of C13orf25

Since the previous experiments revealed an essential role of STAT3 for the induction of C13orf25, as a next step, a screening for STAT3 binding sites in the promoter of C13orf25 was assessed (TFsearch, Computational Biology Research Center, AIST, Japan, www.cbrc.jp/research/db/TFSEARCH.html) and revealed a binding site app. 100 bp upstream of the respective start codon. Alignment of this region with several mammalian species is shown in Figure 4a and underscores the evolutionary conservation of this binding site. To confirm this potential binding site, promoter activity studies were performed. We thus inserted the promoter of C13orf25 upstream to a luciferase reporter construct (pGL3 basic promoter wildtype, WT). In addition, the sequence of the predicted binding site was altered by introduction of three point mutations and used as control (pGL3 basic promoter ΔSTAT3). Transfection was performed in an IL-6 responsive tumor cell line (HepG2). Stimulation of these cells with IL-6 yielded a higher relative luciferase activity of the promoter WT (1.62 ± 0.41) as compared to the mutated promoter construct (ΔSTAT3, 1.04 ± 0.25, p = 0.027, Fig. 4b) indicating the functional importance of this motif in the IL-6 mediated activation of C13orf25 transcription.

Transfection of persistent activated STAT3 downregulates the expression levels of BMPR2 in an in vitro system

To investigate the question whether the activation of STAT3 might affect the protein levels of BMPR2 through changes in the expression levels of miR-17/92, human STAT3 and a constitutively active form of human STAT3, STAT3-C, were cloned.[24] To prevent experimental cross-reaction by overexpression of STAT3, HEK293 cells were used that express endogenous STAT3 in low amounts only. As positive read-out, the mRNA expression of suppressor of cytokine signaling (SOCS3), a well-known target gene of STAT3,[25] was quantified. Overexpression of the wildtype form of STAT3 resulted in increased levels of SOCS3 as compared to mock transfected cells (2.16 ± 0.79). This effect was even enhanced by the introduction of constitutively active STAT3-C (4.52 ± 2.59, Fig. 5a), thus confirming the accurate construction of this molecule. The same samples were further analysed for the expression of miR-20a as a representative of mature miRNAs derived from miR-17/92 (Fig. 5b). Thereby, a similar expression pattern was seen in HEK293 cells for miR-20a as for SOCS3, showing significantly increased miR-20a levels in STAT3 WT transfected cells (1.6 ± 0.24, $p = 0.016$) and STAT3-C transfected cells (2.89 ± 0.63, $p = 0.03$). These data emphasize that the STAT3 pathway modulates the expression of mature miRNAs encoded by the miR-17/92 cluster.

The data presented so far showed that (i) the BMPR2 gene is regulated on a post-transcriptional level by miR-17-5p and miR-20a; and that (ii) the activation of STAT3 upregulates the preliminary transcript C13orf25 and the mature miR-20a. To link these findings, STAT3-C was overexpressed in HEK293 cells and, subsequently, the BMPR2 expression was analysed after 96h (Fig 5c). Analysis of the mRNA levels of BMPR2 revealed no significant changes (0.94 ± 0.45 fold compared to mock) as shown in panel A. Conversely, the protein expression of BMPR2 was found to be reduced by app. 30% after constitutive activation of STAT3 (Fig. 5d).

Discussion

In the present study, we found that (i) the protein expression of BMPR2 is modulated by the miR-17/92 cluster without affecting the BMPR2 mRNA levels; (ii) that this regulatory effect is driven by two distinct miRNAs, i.e. miR-17-5 and miR-20a through conserved seed matches within the 3'UTR of BMPR2; (iii) that IL-6 regulates the expression of the miR-17/92 in human pulmonary arterial endothelial cells by signaling through STAT3. Moreover, we could show that (iiii) the promoter region of C13orf25 exhibits an evolutionary conserved STAT3 binding site; and, finally, (iiiii) that persistent activation of STAT3 leads to a strong upregulation of mature miR-20a, which, in turn, reduces the expression of BMPR2 protein. Taken together, our findings offer a novel mechanistic explanation for the downregulation of BMPR2, which has been repeatedly described as important feature in the pathogenesis of pulmonary hypertension.

The cell surface receptor BMPR2 is essential for the modulation of differentiation, proliferation and the fibrous matrix production of both endothelial and smooth muscle cells.[26,7] Changes in the expression of BMPR2 thus might promote vascular remodelling as observed in the arterial vessels of patients with pulmonary hypertension. This hypothesis is supported by the fact that the loss of BMPR2 due to germline mutations is a hallmark of genetic forms of pulmonary hypertension and, moreover, that reduced BMPR2 levels in the pulmonary arteries have been described in several animal models of pulmonary hypertension.[8,10] The regulation of BMPR2 however is poorly understood so far, but results from previous studies suggest a post-transcriptional mechanism of regulation.[8,10] In this field, miRNAs have emerged as novel molecular players. The data presented here comprise to our knowledge the first report on the modulation of BMPR2 by microRNAs.

We approached the experiments by performing a computational screening that revealed multiple miRNAs as potential regulators of BMPR2. The highest prediction rates however were yielded for the miRNAs derived from the miRNA-17/92 cluster located in the 3rd intron of the C13orf25 gene. It was previously shown that this polycistronic region encodes for six mature microRNAs (i.e. the miR-17/92 cluster: miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92).[18] To investigate the role of these miRNAs in the regulation of BMPR2, we employed a mammalian pcDNA expression vector encoding for miR-17/92. Transient overexpression of this cluster led to a reduction of BMPR2 protein levels, whereas the mRNA levels remained unchanged. These results confirmed the hypothesis of a post-transcriptional role of miR-17/92 in the regulation of BMPR2. To proof whether these miRNAs directly interact with the 3'UTR of BMPR2, we further constructed a reporter gene system containing a luciferase gene and the predicted seed matches for miR-17/92. This assay showed repressed luciferase activity following overexpression of miR-17/92 thus verifying a direct binding of these miRNAs. Conversely, elevated levels of luciferase activity were found after blocking the endogenous miRNAs by using individually designed anti-miRs. In particular, we could show that the 3'UTR of BMPR2 is a direct target of two distinct microRNAs, i.e. miR-17-5p and miR-20a, derived from the miR-17/92 cluster. Consistent with these data, TGFβR2, another receptor from the identical protein family, is also targeted by the miR-17/92 cluster.[13] TGFβR2 is a major mediator of tissue fibrosis and has been associated with the pathogenesis of pulmonary hypertension.[27] Moreover, it has been found that the TGFβR2 protein is almost absent in plexiform lesions that characterize the aberrant endothelial proliferation in idiopathic pulmonary arterial hypertension.[28] Whether the miRNAs derived from the cluster 17/92 regulate TGFβR2 and BMPR2 alike and, thus, might provide a final common pathway in the remodelling of pulmonary arterial vessels has to be addressed by further studies.

The gene C13orf25 was first described as target for chromosomal amplification in malignant lymphoma.[29] This gene attracted primary attention after several studies suggested oncogenic activities probably due to targeting tumor suppressor genes, such as Bim and PTEN.[30] Moreover, Suarez and co-workers showed that the miR-17/92 cluster is pivotally involved in the angiogenic sprouting of human endothelial cells.[31] Recent observations demonstrated that the expression pattern of miRNAs derived from miR-17/92 is regulated by a number of known transcription factors. O'Donnell and co-workers, for example, described the modulation of miR-17/92 by c-Myc[14] and the data on this regulatory network have recently been extended by Woods et al. showing a direct interaction of E2F3 with the promoter region of C13orf25.[32]

In our experiments, we provide evidence that the promoter of C13orf25 also bears a functional binding site for STAT3, which controls the transcription of several genes involved in the inflammatory response.[33] By applying promoter studies we confirmed a STAT3-responsive region located about 100 bp upstream of the start codon of C13orf25. The functional importance of these findings is highlighted by the fact that this region was found to be phylogenetically conserved among mammalian species. In all species investigated, the distance of the STAT3-binding site to the respective sequence encoding for miR-17/92 revealed to span between 3200 and 4500 bp. Interestingly, a similar distance was described recently for the other known STAT3-regulated miRNA (i.e. miR-21),[20] indicating a common evolutionary assembly of miRNA genes regulated by this transcription factor.

In normal cells, the expression and phosphorylation of STAT3 is finely balanced by negative feedback loops including the activation of SOCS proteins.[25] These feedback mechanisms might however be bypassed through persistent upstream signaling or through knock-down of inhibitory proteins leading to constitutively activated STAT3. Such phenomena have been found in several human tumors.[34] Of interest, a constitutive activation of STAT3 has also been described in human arterial endothelial cells derived from patients with pulmonary

hypertension.[35] In general, the imitation of aberrant signaling by overexpression of persistently activated STAT3 displays an interesting experimental approach to identify novel miRNAs regulated directly or indirectly through inflammatory responses. The results obtained by such strategies might help to understand the reasons for altered miRNA expression profiles in these conditions. Along this line, we constructed such an expression vector for STAT3,[24] and subsequent transfection of this vector promptly resulted in increased levels of mature miR-20a as compared to mock or STAT3 wildtype transfected cells. Since this experimental setup reduced the expression of BMPR2 protein but not the respective mRNA, we suggest that this effect is driven, at least in part, by the upregulation of miRNAs derived from the cluster miR-17/92.

STAT3 was first described in the downstream signaling of IL-6 modulating acute phase gene expression.[36] Intriguingly, patients with pulmonary hypertension were found to have higher serum levels of IL-6 as compared to healthy controls[3] and the ectopic administration of IL-6 has been observed to induce a mild elevation of the pulmonary arterial pressure in mice.[37,38] Moreover, the important role of IL-6 in the pathogenesis of pulmonary hypertension has been underpinned by a recent study in transgenic mice overexpressing IL-6. When compared to their wildtype counterparts, these animals developed increased ventricular systolic pressures, right ventricular hypertrophy and pulmonary vasculopathic changes indicative for pulmonary hypertension.[5] In the present study, we thus investigated the influence of IL-6 on the expression of C13orf25 in HPAEC, and found that IL-6 induced the gene expression of miR-17/92 in a STAT3-dependent manner.

Our data offer a direct link between the action of IL-6 and the expression of the miR-17/92 cluster. Since STAT3 has been shown to activate also the transcription of the c-myc gene,[39]

our findings highlight the role of IL-6 in the regulatory pathway that controls the expression of miR-17/92 and thus complements this network by a novel piece (Figure 6).

Finally, our data allow us to conclude that increased IL-6 signaling leads to the downregulation of BMPR2, based on a phylogenetically conserved STAT3 - miR-17/92 pathway. It could be speculated that STAT3 plays an important role in the development of pulmonary hypertension, in particular since it has been shown that a persistently activated STAT3 promotes cell survival of HPAEC derived from patients with pulmonary arterial hypertension. As we could further show that STAT3 regulates the BMPR2 expression through transcriptional activation of miR-17/92, one might postulate this cluster as a highly specific target for the causative treatment of pulmonary hypertension. Since inhibitors of miRNAs are not available in a clinical setting at this moment, the inhibition of STAT3 activation by anti-cytokine therapies directed against IL-6 might provide a feasible alternative to restore functional levels of BMPR2.

Taken together, we provide here for the first time a mechanistic explanation for the loss of BMPR2 in pulmonary hypertension shedding novel light on the pathogenesis of this disease and related conditions.

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Disclosures

None.

References

1. Humbert M, Sitbon O, Simonneau G. Treatment of pulmonary arterial hypertension. *N Engl J Med.* 2004;351:1425-1436.

2. Farber HW, Loscalzo J. Pulmonary arterial hypertension. *N Engl J Med.* 2004;351:1655-1665.

3. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G, Emilie D. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *Am J Respir Crit Care Med.* 1995;151:1628-1631.

4. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol.* 2004;43:13S-24S.

5. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB. Interleukin-6 Overexpression Induces Pulmonary Hypertension. *Circ Res.* 2008.

6. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest.* 2008;118:2372-2379.

7. Zhang S, Fantozzi I, Tigno DD, Yi ES, Platoshyn O, Thistlethwaite PA, Kriett JM, Yung G, Rubin LJ, Yuan JX. Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. *American journal of physiology.* 2003;285:L740-754.

8. Takahashi H, Goto N, Kojima Y, Tsuda Y, Morio Y, Muramatsu M, Fukuchi Y. Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2006;290:L450-458.

9. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation.* 2002;105:1672-1678.

10. Morty RE, Nejman B, Kwapiszewska G, Hecker M, Zakrzewicz A, Kouri FM, Peters DM, Dumitrascu R, Seeger W, Knaus P, Schermuly RT, Eickelberg O. Dysregulated bone morphogenetic protein signaling in monocrotaline-induced pulmonary arterial hypertension. *Arteriosclerosis, thrombosis, and vascular biology.* 2007;27:1072-1078.

11. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 2005;120:15-20.

12. Mendell JT. MicroRNAs: critical regulators of development, cellular physiology and malignancy. *Cell Cycle.* 2005;4:1179-1184.

13. Tagawa H, Karube K, Tsuzuki S, Ohshima K, Seto M. Synergistic action of the microRNA-17 polycistron and Myc in aggressive cancer development. *Cancer Sci.* 2007;98:1482-1490.

14. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature.* 2005;435:839-843.

15. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* 2005;33:e179.

16. Kuhn DE, Martin MM, Feldman DS, Terry AV, Jr., Nuovo GJ, Elton TS. Experimental validation of miRNA targets. *Methods.* 2008;44:47-54.

17. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. *Nature.* 2008;455:58-63.

18. Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res.* 2005;65:9628-9632.

19. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature.* 2005;433:769-773.

20. Loffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermuller J, Kretzschmar AK, Burger R, Gramatzki M, Blumert C, Bauer K, Cvijic H, Ullmann AK, Stadler PF, Horn F. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood.* 2007;110:1330-1333.

21. Sun Q, Zhang Y, Yang G, Chen X, Zhang Y, Cao G, Wang J, Sun Y, Zhang P, Fan M, Shao N, Yang X. Transforming growth factor-beta-regulated miR-24 promotes skeletal muscle differentiation. *Nucleic acids research.* 2008;36:2690-2699.

22. Ni CW, Hsieh HJ, Chao YJ, Wang DL. Interleukin-6-induced JAK2/STAT3 signaling pathway in endothelial cells is suppressed by hemodynamic flow. *Am J Physiol Cell Physiol.* 2004;287:C771-780.

23. Bartoli M, Gu X, Tsai NT, Venema RC, Brooks SE, Marrero MB, Caldwell RB. Vascular endothelial growth factor activates STAT proteins in aortic endothelial cells. *J Biol Chem.* 2000;275:33189-33192.

24. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr. Stat3 as an oncogene. *Cell.* 1999;98:295-303.

25. Krebs DL, Hilton DJ. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells.* 2001;19:378-387.

26. Reddi AH. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat Biotechnol.* 1998;16:247-252.

27. Eickelberg O, Morty RE. Transforming growth factor beta/bone morphogenic protein signaling in pulmonary arterial hypertension: remodeling revisited. *Trends Cardiovasc Med.* 2007;17:263-269.

28. Yeager ME, Halley GR, Golpon HA, Voelkel NF, Tuder RM. Microsatellite instability of endothelial cell growth and apoptosis genes within plexiform lesions in primary pulmonary hypertension. *Circ Res.* 2001;88:E2-E11.

29. Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S, Yoshida Y, Seto M. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res.* 2004;64:3087-3095.

30. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. *Cell.* 2008;133:217-222.

31. Suarez Y, Fernandez-Hernando C, Yu J, Gerber SA, Harrison KD, Pober JS, Iruela-Arispe ML, Merkenschlager M, Sessa WC. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. *Proc Natl Acad Sci U S A.* 2008;105:14082-14087.

32. Woods K, Thomson JM, Hammond SM. Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. *J Biol Chem.* 2007;282:2130-2134.

33. Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol.* 2002;3:651-662.

34. Turkson J, Jove R. STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene.* 2000;19:6613-6626.

35. Masri FA, Xu W, Comhair SA, Asosingh K, Koo M, Vasanji A, Drazba J, Anand-Apte B, Erzurum SC. Hyperproliferative apoptosis-resistant endothelial cells in

idiopathic pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2007;293:L548-554.

36. Wegenka UM, Buschmann J, Lutticken C, Heinrich PC, Horn F. Acute-phase response factor, a nuclear factor binding to acute-phase response elements, is rapidly activated by interleukin-6 at the posttranslational level. *Mol Cell Biol.* 1993;13:276-288.

37. Golembeski SM, West J, Tada Y, Fagan KA. Interleukin-6 causes mild pulmonary hypertension and augments hypoxia-induced pulmonary hypertension in mice. *Chest.* 2005;128:572S-573S.

38. Savale L, Tu L, Rideau D, Izziki M, Maitre B, Adnot S, Eddahibi S. Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice. *Respir Res.* 2009;10:6.

39. Kiuchi N, Nakajima K, Ichiba M, Fukada T, Narimatsu M, Mizuno K, Hibi M, Hirano T. STAT3 is required for the gp130-mediated full activation of the c-myc gene. *J Exp Med.* 1999;189:63-73.

Figure legends

Figure 1:

Overexpression of miR-17/92 in HEK293 cells.

HEK293 cells were transfected with the pcDNA miR-17/92 vector, and mRNA and protein were collected 72h later. A) Quantification of miR-20a showed a significant upregulation by 2.71 ± 0.45 fold as compared to mock transfection. B) Transient overexpression of the miR-17/92 cluster led to a 40% decrease of BMRP2 protein as analysed by Western blot. C) Levels of BMPR2 mRNA were slightly reduced (0.89 ± 0.06). Data are shown as mean ± SD derived from 4 independent experiments.

Figure 2:

Reporter gene studies on the interaction between 3'UTR of BMPR2 and the miR-17/92 cluster in HEK293 cells.

A) Co-transfection of the pGL3 3'UTR of BMPR2 "sense" construct and the miR-17/92 overexpressing vector resulted in a significantly lower relative luciferase activity as compared to the "anti-sense" construct ($p = 0.0047$). B) Blocking experiments with the use of anti-sense RNA molecules (anti-miRs) directed against each miRNA revealed a significantly higher relative luciferase activity for anti-miR-17-5p and anti-miR-20a. Data are shown as mean ± SD derived from 4 independent experiments.

Figure 3:

Correlation between the expression of miR-17/92 and the activity of STAT3 in HPAEC.

A) Quantification of the preliminary transcript of miR-17/92 (C13orf25). Stimulation of HPAEC with IL-6 and VEGF but not PDGF significantly increased the expression of C13orf25 mRNA. B) Phosphorylation of STAT3 at tyrosine residue 705 could be observed

for IL-6 in a time dependent manner, whereas no effect was seen for the stimulation with VEGF. C) siRNA-mediated knockdown reduced the expression of STAT3 by 50% on the protein level. D) The induction of C13orf25 mRNA by IL-6 was almost completely abolished by the functional knockdown of STAT3 as compared to stimulated scrambled control cells ($p = 0.0027$). The effect observed by stimulation with VEGF was less strong but still reached statistical significance ($p = 0.0428$). E) Stimulation of HPAECs for 24h revealed significant upregulation of miR-20a after stimulation with IL-6 ($p = 0.033$). Data are shown as mean ± SD derived from 4 independent experiments.

Figure 4:

Identification of a highly conserved STAT3 binding site in the promoter of C13orf25.

A) A 33 bp region in the promoter of C13orf25 contains a predicted STAT3 binding site. Alignment of this region with several mammalian species highlights the evolutionary conservation of this binding site (adapted from [20]). B) Reporter gene assay studies. Stimulation of HepG2 cells with IL-6 resulted in a higher relative luciferase activity of the promoter WT as compared to the mutated promoter construct (ΔSTAT3). Data are blotted as median including lower and upper whisker. 5 independent experiments performed.

Figure 5:

Transient transfection of constitutively active STAT3 downregulates BMPR2 in vitro.

A) Quantification of SOCS3 mRNA in transfected HEK293 cells after 48h. Overexpression of both STAT3-WT and STAT3-C resulted in increased levels of SOCS3 as compared to mock transfected cells. B) Analysis of miR-20a expression performed in identical RNA extracts as in panel A. Levels of miR-20a were found upregulated in STAT3-WT and STAT3-C transfected cells when compared to mock transfected cells. C) Quantification of BMPR2 mRNA in STAT3-C transfected HEK293 cells after 96h. The mRNA levels of BMPR2 was

found unchanged as compared to mock. D) Western blot for BMPR2 in STAT3-C transfected HEK293 cells after 96h showed a reduction of protein expression. Data are shown as mean ± SD derived from 4 independent experiments.

Figure 6:

Mechanism of regulation of BMPR2.

The transcription of miR-17/92 is regulated by c-Myc and E2F3.[14,32] In the present study, we demonstrate that the promoter region of miR-17/92 also bears a functional binding site for STAT3. The latent transcription factor STAT3 is the major mediator of IL-6 signaling. With respect to the fact that STAT3 induces the expression of c-Myc,[39] IL-6 emerges as a new modulator of miR-17/92. It is known that microRNAs derived from this cluster target the surface receptor TGFβR2.[13] Here we provide evidence that the related BMPR2 is modulated by miR-17/92 alike.

Figure 1

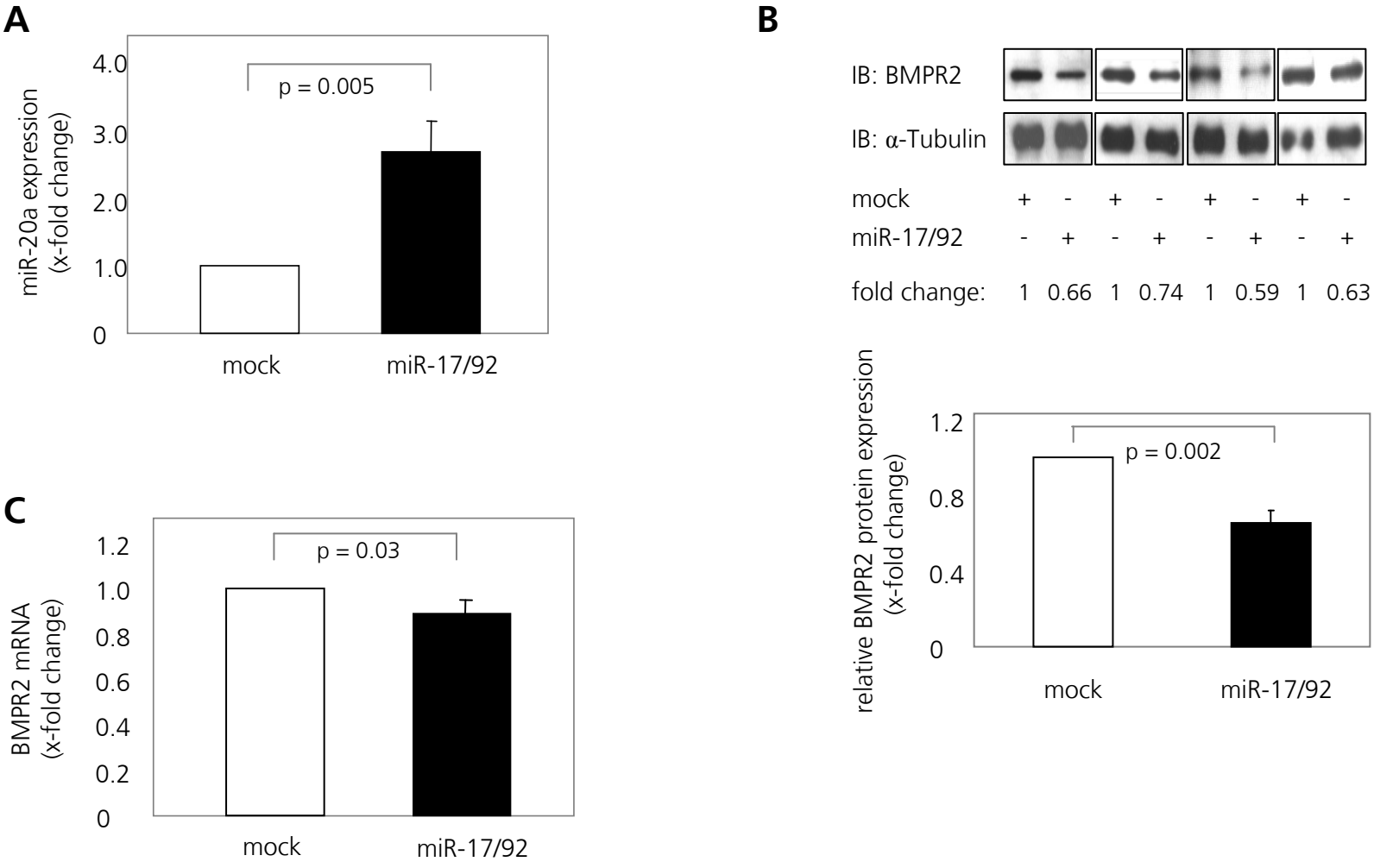
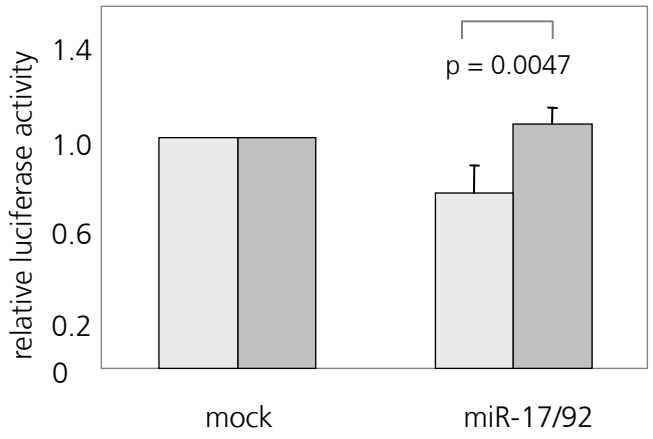
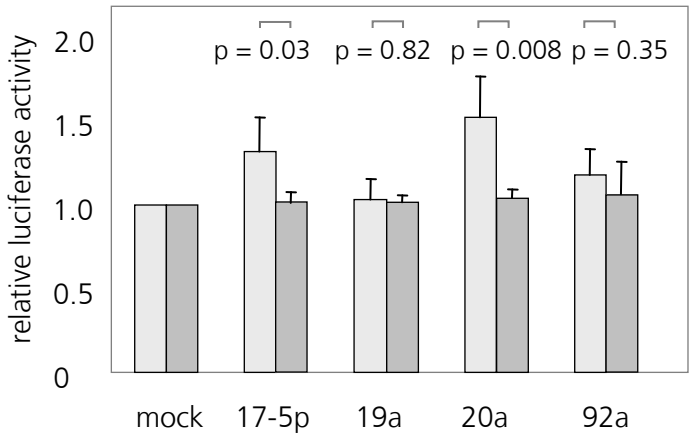

A
miR-20a expression (x-fold change)
4.0
3.0
2.0
1.0
0
p = 0.005
mock
miR-17/92
B
IB: BMPR2
IB: α-Tubulin
mock + - + - + - + -
miR-17/92 - + - + - + - +
fold change: 1 0.66 1 0.74 1 0.59 1 0.63
relative BMPR2 protein expression (x-fold change)
1.2
0.8
0.4
0
p = 0.002
mock
miR-17/92
C
BMPR2 mRNA (x-fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0
p = 0.03
mock
miR-17/92

Figure 2

A



B



pGL3 control 3' UTR of BMPR2 5' to 3' („sense")
pGL3 control 3' UTR of BMPR2 3' to 5' („anti-sense")

pGL3 control 3' UTR of BMPR2 5' to 3' („sense")
pGL3 control 3' UTR of BMPR2 3' to 5' („anti-sense")

Figure 3

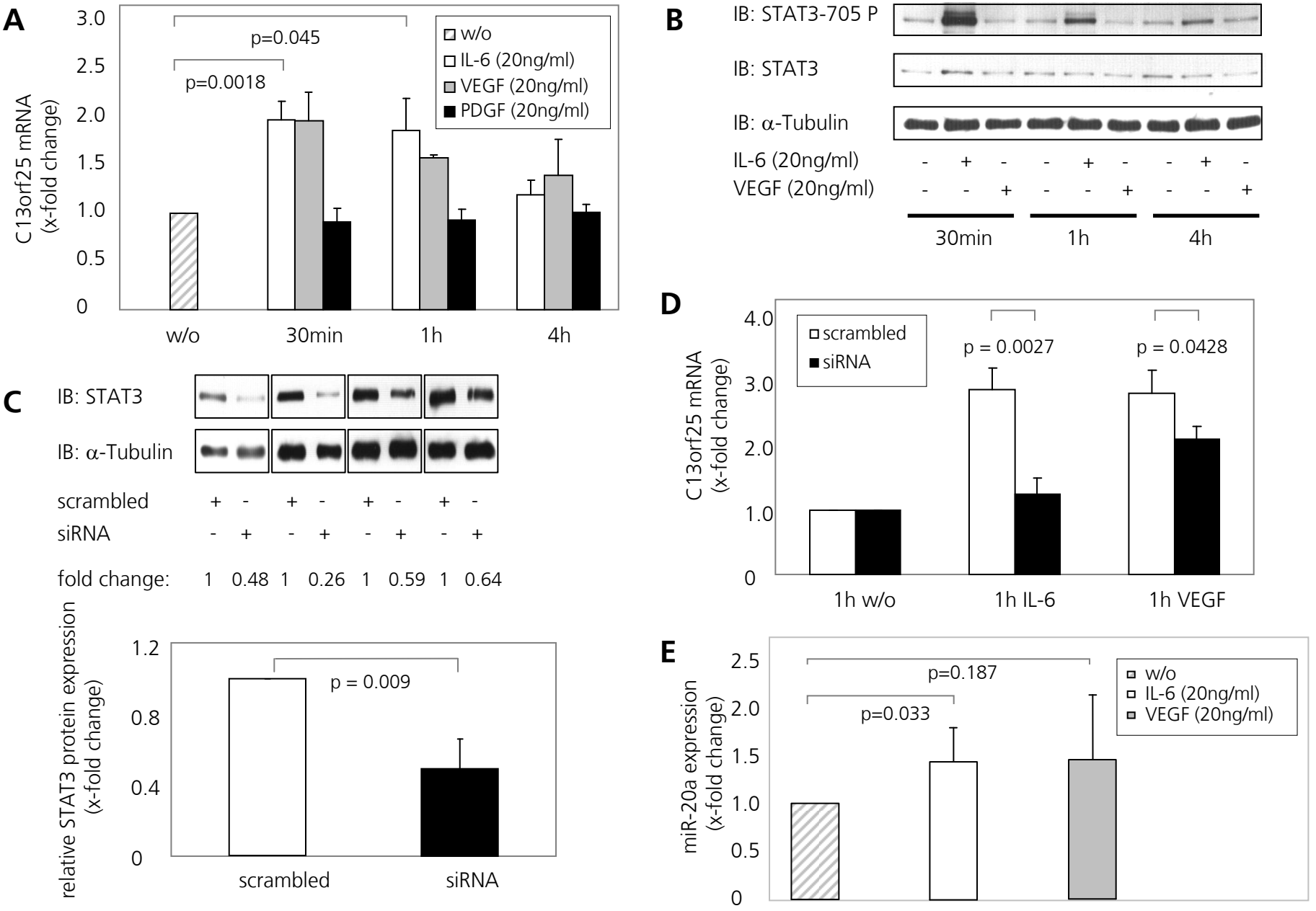
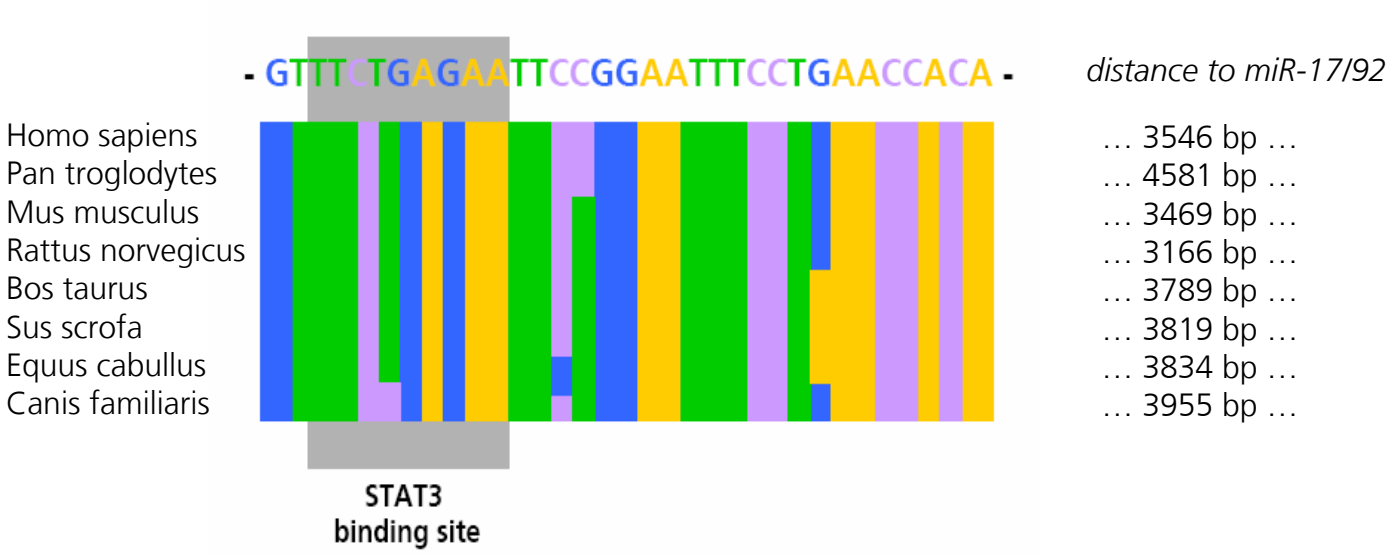

A
C13orf25 mRNA (x-fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0
p=0.045
p=0.0018
w/o
IL-6 (20ng/ml)
VEGF (20ng/ml)
PDGF (20ng/ml)
w/o
30min
1h
4h
B
IB: STAT3-705 P
IB: STAT3
IB: α-Tubulin
IL-6 (20ng/ml)
VEGF (20ng/ml)
30min
1h
4h
C
IB: STAT3
IB: α-Tubulin
scrambled
siRNA
fold change: 1 0.48 1 0.26 1 0.59 1 0.64
relative STAT3 protein expression (x-fold change)
1.2
0.8
0.4
0
p = 0.009
scrambled
siRNA
D
C13orf25 mRNA (x-fold change)
4.0
3.0
2.0
1.0
0
scrambled
siRNA
p = 0.0027
p = 0.0428
1h w/o
1h IL-6
1h VEGF
E
miR-20a expression (x-fold change)
2.5
2.0
1.5
1.0
0.5
0
p=0.187
p=0.033
w/o
IL-6 (20ng/ml)
VEGF (20ng/ml)

Figure 4

A


- GTTTCTGAGAATTCCGGAATTTCCTGAACCACA -
distance to miR-17/92
Homo sapiens
Pan troglodytes
Mus musculus
Rattus norvegicus
Bos taurus
Sus scrofa
Equus cabullus
Canis familiaris
… 3546 bp …
… 4581 bp …
… 3469 bp …
… 3166 bp …
… 3789 bp …
… 3819 bp …
… 3834 bp …
… 3955 bp …
STAT3
binding site

B

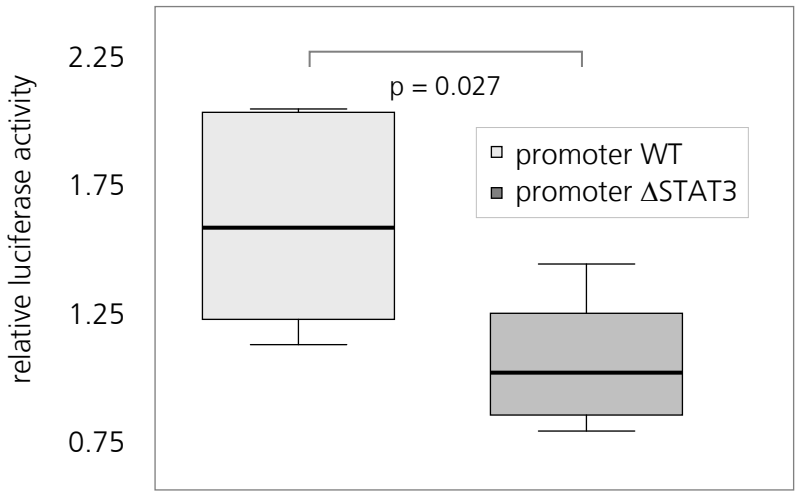

relative luciferase activity
2.25
1.75
1.25
0.75
p = 0.027
promoter WT
promoter ΔSTAT3

Figure 5

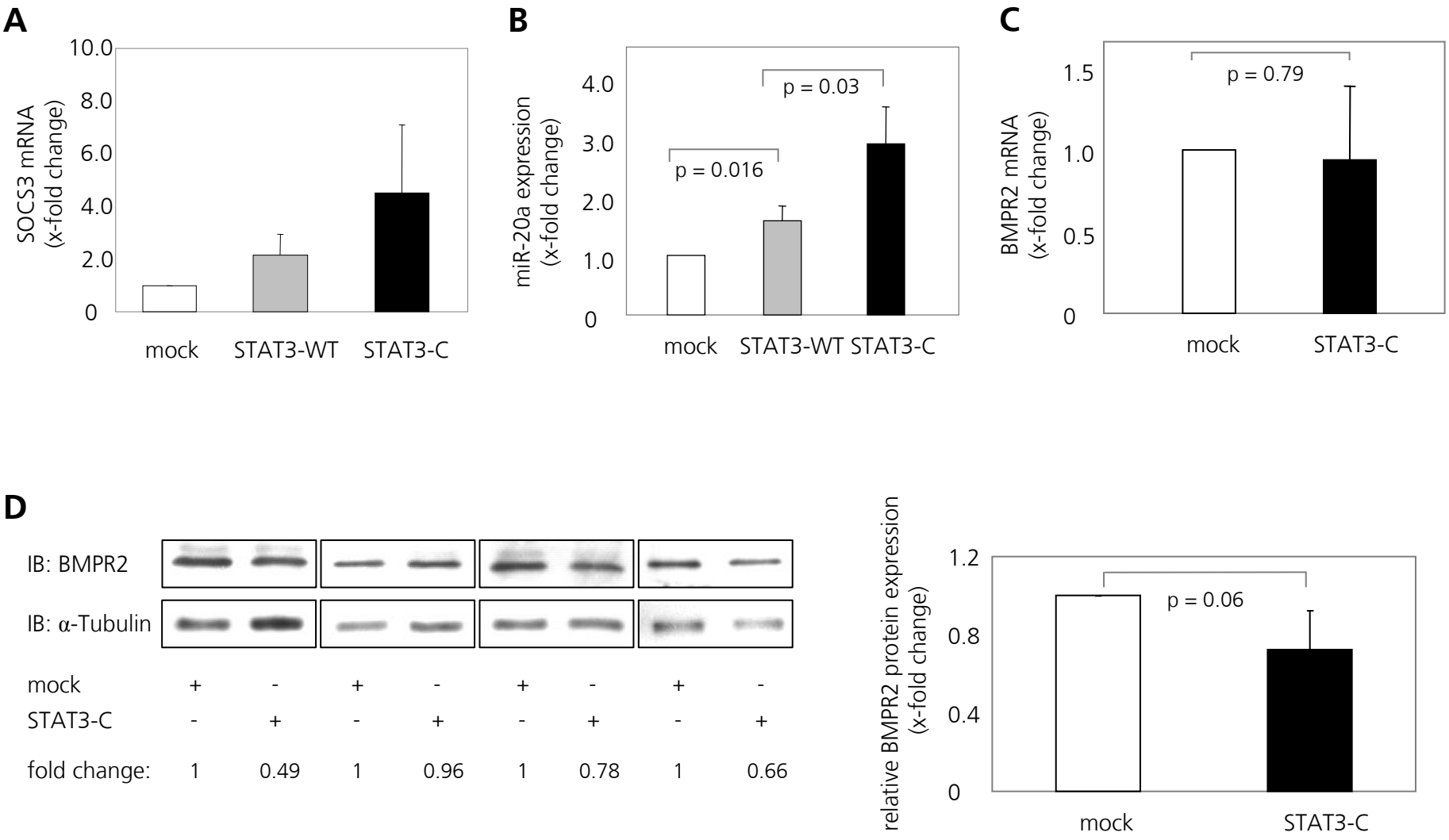

A
SOCS3 mRNA (x-fold change)
10.0
8.0
6.0
4.0
2.0
0
mock
STAT3-WT
STAT3-C
B
miR-20a expression (x-fold change)
4.0
3.0
2.0
1.0
0
p = 0.016
p = 0.03
mock
STAT3-WT
STAT3-C
C
BMPR2 mRNA (x-fold change)
1.5
1.0
0.5
0
p = 0.79
mock
STAT3-C
D
IB: BMPR2
IB: α-Tubulin
mock + - + - + - + -
STAT3-C - + - + - + - +
fold change: 1 0.49 1 0.96 1 0.78 1 0.66
relative BMPR2 protein expression (x-fold change)
1.2
0.8
0.4
0
p = 0.06
mock
STAT3-C

Figure 6